Inhibition of arterial medial calcification and bone mineralization by extracellular nucleotides

Inhibition of arterial medial calcification and bone mineralisation by extracellular nucleotides: the same functional effect mediated by different cellular mechanisms

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ABSTRACT

Arterial medial calcification (AMC) is thought to share some outward similarities to skeletal mineralisation and has been associated with the transdifferentiation of vascular smooth muscle cells (VSMCs) to an osteoblast-like phenotype. ATP and UTP have previously been shown to inhibit bone mineralisation. This investigation compared the effects of extracellular nucleotides on calcification in VSMCs with those seen in osteoblasts. ATP, UTP and the ubiquitous mineralisation inhibitor, pyrophosphate (PP_i), dose dependently inhibited VSMC calcification by ≤85%. Culture of VSMCs in calcifying conditions was associated with an increase in apoptosis; treatment with ATP, UTP and PP_i reduced apoptosis to levels seen in non-calcifying cells. Extracellular nucleotides had no effect on osteoblast viability. Basal alkaline phosphatase (TNAP) activity was over 100-fold higher in osteoblasts than VSMCs. ATP and UTP reduced osteoblast TNAP activity (≤50%) but stimulated VSMC TNAP activity (≤88%). The effects of extracellular nucleotides on VSMC calcification, cell viability and TNAP activity were unchanged by deletion or inhibition of the $P2Y_2$ receptor. Conversely, the actions of ATP/UTP on bone mineralisation and TNAP activity were attenuated in osteoblasts lacking the $P2Y_2$ receptor. Ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (NPP1) hydrolyses ATP and UTP to produce PP_i. In both VSMCs and osteoblasts, deletion of NPP1 blunted the inhibitory effects of extracellular nucleotides suggesting involvement of P2 receptor independent pathways. Our results show that although the overall functional effect of extracellular nucleotides on AMC and bone mineralisation is similar there are clear differences in the cellular mechanisms mediating these actions.

INTRODUCTION

Vascular calcification is a common consequence of ageing, atherosclerosis, diabetes and chronic kidney disease. It is the pathological deposition of calcium phosphate mineral, usually as hydroxyapatite, in the medial and/or intimal layer of the arteries and heart valves. Arterial medial calcification (AMC) refers to the calcification that occurs within the tunica media of blood vessels and is characterised by increased vessel stiffness and reduced blood flow (Young et al., 1993). Traditionally, AMC was thought to be a passive process caused by high serum levels of phosphate and calcium. However, it is now accepted that it is a complex cell-mediated process that shares some similarities with the process of physiological bone formation. Specifically, AMC is thought to involve the loss of calcification inhibitors (e.g. pyrophosphate (PP_i), osteopontin, fetuin A), gain of calcification inducers (e.g. alkaline phosphatase (TNAP)) and increased apoptosis (Proudfoot et al., 2000; Zhu et al., 2012). Whilst many cell types can contribute towards the development of AMC, vascular smooth muscle cells (VSMCs) are thought to be the major cell type involved (Narisawa et al., 2007; Zhu et al., 2011). Within a calcifying environment (high phosphate), VSMCs can undergo a phenotypic transdifferentiation to take on characteristics usually associated with bone-forming osteoblasts (Shroff and Shanahan, 2007; Zhu et al., 2011).

ATP has long been recognized for its role in intracellular energy metabolism; however, it is also an important extracellular signalling molecule. ATP and related compounds (UTP, ADP, UDP) act via purinergic P2 receptors to regulate cell proliferation, differentiation, survival and function in many tissues (Burnstock, 2007). The P2 receptor family is made up of seven P2X ion channels (P2X1-7) and eight P2Y G-protein coupled receptors ($P2Y_{1,2,4,6,11-14}$) (Abbracchio and Burnstock, 1994; Burnstock and Kennedy, 1985).

Purinergic signalling plays a number of roles in the cardiovascular system (see review (Burnstock and Ralevic, 2014)). VSMCs express multiple P2 receptor subtypes (Wang et al., 2002) and, in recent years, several investigations have examined the role of purinergic signalling in the different forms of vascular calcification (Fish et al., 2013). However, they provide conflicting evidence as to whether extracellular nucleotides are harmful or protective. $P2Y_2$ receptor mediated signalling has been shown to promote the survival of aortic valve interstitial cells and protect against aortic valve calcification (Cote et al., 2012) and arterial intimal calcification (Qian et al., 2017). Additionally, a biochemical study showed that ATP inhibited calcium phosphate deposition in rat VSMCs (Villa-Bellosta and Sorribas, 2013). In contrast, activation of P2YRs by Up_4A (a non-selective P2R agonist) has been reported to enhance VSMC calcification (Schuchardt et al., 2012).

Once released extracellular nucleotides are rapidly broken down by ecto-nucleotidases to limit their actions to cells within close proximity of the release site. PP_i, a ubiquitous and potent inhibitor of calcification (Fleisch and Bisaz, 1962), is generated when nucleotide triphosphates are hydrolysed by ecto-nucleotide pyrophosphatase/phosphodiesterases (NPPs). VSMCs express many ecto-nucleotidases, including NPP1, and can release ATP in a controlled manner (Prosdocimo et al., 2009; Prosdocimo et al., 2010; Villa-Bellosta et al., 2011). Accumulating evidence now suggests that the hydrolysis of locally released ATP by NPP1 is the major source of extracellular PP_i (Orriss et al., 2016; Prosdocimo et al., 2009). This PP_i can then act locally to regulate the level of calcification. Consistent with this, NPP1 knockout mice (*Enpp1*$^{-/-}$) display extensive ectopic calcification in a variety of soft tissues including the aorta, kidney, cartilage, ear pinna and whisker vibrissae (Hajjawi et al., 2014; Johnson et al., 2003; Mackenzie et al., 2012). Furthermore, mutations in the gene encoding NPP1 lead to the recessive condition Generalised Arterial Calcification of Infancy (GACI) which is characterised by extensive vascular calcification (Rutsch et al., 2001).

The regulation of bone formation by extracellular nucleotides has been widely studied often with conflicting results (see reviews (Burnstock et al., 2013; Gartland et al., 2012; Noronha-Matos and Correia-de-Sa, 2016; Orriss, 2015)). Activation of several P2 receptors (e.g. $P2Y_2$, P2X1, P2X7) has been shown to both inhibit and promote bone mineralisation (Hoebertz et al., 2002; Noronha-Matos et

al., 2014; Orriss et al., 2012a; Orriss et al., 2007; Panupinthu et al., 2007; Xing et al., 2014). We have previously reported that ATP and UTP selectively inhibit the mineralisation of the organic matrix and TNAP expression and activity (Orriss et al., 2007). These actions are due to both P2 receptor mediated signalling and also direct hydrolysis by NPP1 to produce PP_i (Orriss et al., 2012a; Orriss et al., 2013; Orriss et al., 2007). Recently, we also demonstrated that activation of the $P2Y_2$ receptor exerts some of its effects on bone mineralisation indirectly by promoting the release of ATP from osteoblasts (Orriss et al., 2017). In contrast to the potent actions of ATP and UTP, ADP and UDP do not influence bone mineralisation (Orriss et al., 2007).

The aim of this study was to investigate the effects of extracellular nucleotides on AMC and, given the apparent similarities to bone mineralisation, to compare any functional effects to those seen in osteoblasts. Established *in vitro* mouse models of AMC and bone formation were used to determine the cellular mechanisms mediating these actions. Since the inhibitory effects of ATP and UTP on bone mineralisation involve both P2 receptor dependent and independent signalling we examined both pathways in parallel.

METHODS

Reagents

All tissue culture reagents were purchased from Life Technologies (Paisley, UK); unless mentioned, all chemicals were purchased from Sigma Aldrich (Poole, UK). The selective $P2Y_2$ receptor antagonist, ARC118935XX, was obtained from Tocris Bioscience (Bristol, UK).

Animals

Mice lacking the $P2Y_2$ receptor gene (*$P2Y_2R^{-/-}$*) were obtained from Jackson Laboratories (Bar Harbor, Maine, USA). The generation and characterisation of *$P2Y_2R^{-/-}$* mice, which are on a C57BL/6J background, has been previously described (Homolya et al., 1999). Animals were bred from homozygote (*$P2Y_2R^{-/-}$*) and parental strain wildtype (*$P2Y_2R^{+/+}$*) breeding pairs. The generation and characterisation of mice lacking NPP1 (*$Enpp1^{-/-}$*), which are on a 129Sv/TerJ genetic background, has previously been described (Sali et al., 1999). Animals were bred from heterozygote (*$Enpp1^{+/-}$*) breeding pairs due to the inability of homozygotes to breed. All mice were housed under standard conditions with free access to food and water. All procedures complied with the UK animals (Scientific Procedures) Act 1986 and were reviewed and approved by the Royal Veterinary College Research Ethics Committee.

Vascular smooth muscle cell (VSMC) calcification assay

Primary VSMCs were isolated from aortas of *$P2Y_2R^{-/-}$* or *$Enpp1^{-/-}$* mice and their corresponding wildtypes (*$P2Y_2R^{+/+}$ or $Enpp1^{+/+}$*). After removal of the adventitia, the aorta was opened to expose the endothelial layer under a dissection microscope. Tissues from 6-8 animals were pooled and incubated with trypsin (0.25% *w/v*) for 10 minutes to remove any remaining adventitia and endothelium. Tissues were incubated overnight in alpha Minimum Essential Medium, supplemented with 10% foetal calf serum (FCS), 100U/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml amphotericin (complete mixture abbreviated to *α*MEM) before being digested with 425U/ml collagenase type II (Worthington Biomedical Corporation, Lakewood, USA) for 5 hours. Isolated VSMCs were expanded in T25 tissue culture flasks in a humidified atmosphere of 5% CO_2-95% air at 37°C until confluent. Following seeding into 24-well plates at a density of 2.5×10^4 cells/well, VSMCs were cultured in control (*α*MEM only) or calcifying medium (*α*MEM + 2mM sodium phosphate) for up to 14 days, with half medium changes every 3 days. Cells were treated with 1-100µM extracellular nucleotides (ATP, ADP, UTP, UDP) or pyrophosphate (PP_i) for the duration of the culture; fresh nucleotide was added at each medium change. The selective $P2Y_2$ receptor antagonist, ARC118935XX, was used to confirm results obtained with *$P2Y_2R^{-/-}$* cells.

Aortic ring calcification assay

Aortas were isolated from *$P2Y_2R^{+/+}$* or *$P2Y_2R^{-/-}$* mice and the adventitia layer removed. The vessels were cut into 2-3mm rings and cultured overnight in serum free αMEM. After 24 hours, the rings were transferred to calcification medium (αMEM plus 2.5mM phosphate and 2.7mM calcium chloride). Aortic rings were cultured for a further 9 days with half medium changes every 3 days.

Determination of VSMC and aortic ring calcification

Calcifying VSMCs or aortic rings were washed twice with phosphate buffered saline (PBS) and incubated with 0.6M HCl at room temperature for 24 hours. Calcium content was measured colorimetrically by stable interaction with *o*-cresolphthalein using a commercially available kit (Sigma-Adrich, Poole, UK) and corrected for total protein concentration using the Bradford assay. Calcium deposition was visualised by alizarin red staining of VSMC cell layers as previously described (Taylor et al., 2014).

Osteoblast bone formation assay

Osteoblasts were isolated from the calvariae of 3-5 day old mice by trypsin/collagenase digestion as previously described (Orriss et al., 2014; Orriss et al., 2012b; Taylor et al., 2014). Cells were obtained from *$P2Y_2R^{-/-}$, $P2Y_2R^{+/+}$, $Enpp1^{-/-}$* or *$Enpp1^{+/+}$* animals. Following isolation, cells were resuspended in

αMEM and cultured for 2-4 days in a humidified atmosphere of 5% CO_2-95% air at 37°C in 75 cm^2 flasks until fully confluent. Cells were sub-cultured into 6-well trays in αMEM supplemented with 2mM β-glycerophosphate and 50μg/ml ascorbic acid, with half medium changes every 3 days. Cells were treated with 1-100μM ATP, UTP or PP_i for the duration of the culture; fresh nucleotide was added at each medium change.

To assess bone formation, experiments were terminated by fixing the cells in 2.5% glutaraldehyde for 5 minutes. Cell culture plates were imaged at 800 dpi using a flat-bed scanner (Epson Perfection 4990 Photo) and the total area of bone nodules formed was quantified by image analysis, as described previously (Orriss et al., 2012b). Cell layers were stained with alizarin red for microscopy.

Analysis of P2 receptor gene expression by real time PCR

VSMCs were cultured in control or calcification medium for 14 days. Osteoblasts were cultured until the onset of mineralisation (14 days). RNA was extracted using RNeasy total RNA (Qiagen Ltd, Crawley, UK), according to the manufacturer's instructions. RNA was quantified and reverse transcribed as previously described (Mackenzie et al., 2014). Levels of mRNA expression were measured using the SYBR green detection method (Roche, East Sussex, UK) as previously reported (Staines et al., 2014). Data are presented as (1) the fold change in expression in calcifying VSMCs relative to control cells and (2) the fold change in expression in calcifying VSMCs compared to mineralising osteoblasts. Primer sequences are shown in table 1.

Cell viability assay

VSMCs and osteoblasts were cultured for 14 days in medium supplemented with ATP, UTP, ADP, UDP or PP_i (1-100μM); fresh nucleotide was added at each medium change. Cell number and viability was determined using the CytoTox 96® colorimetric cytotoxicity assay (Promega UK, Southampton UK), as described previously (Orriss et al., 2012a). Cell supernatants were collected to determine medium LDH levels (cell viability). To establish total cellular LDH levels, cells were lysed with 1% Triton X-100 in water (lysis buffer, 15μl/ml of medium) for 1 hour. The LDH content of the supernatants and cell lysates were measured colorimetrically (490nm) as per manufacturer's instructions. A standard curve for determination of cell numbers was constructed using cells seeded at 10^2 to 10^6/well. Cell viability (shown as percentage of dead cells) was calculated by expressing medium LDH as a percentage of the total cellular LDH.

Quantification of apoptosis by flow cytometry

VSMCs plated in 24-well trays were cultured in control or calcification medium (± treatment) for 7 days. Apoptosis was assessed via flow cytometry using an annexin V antibody conjugated to fluorescein (Life Technologies, Paisley, UK), as per manufacturer's instructions. Briefly, cells were detached using trypsin (0.25%) and resultant pellet washed in ice cold PBS. This suspension was centrifuged and resuspended in 1X annexin-binding buffer (Life Technologies, Paisley, UK). A sample of this suspension was incubated with the annexin V antibody for 15 minutes, after which, analysed using a BD FACSCanto II Flow Cytometer (Becton, Dickinson and Company, Oxford, UK). Data was processed to calculate percentage apoptosis using Flowing Software (version 2.5.1) (Turku University, Finland).

Determination of TNAP activity

VSMCs and osteoblasts VSMCs were cultured with 1-100μM ATP, UTP or PP_i for 14 days; fresh nucleotide was added at each medium change. TNAP activity was measured in *$P2Y_2R^{-/-}$, $Enpp1^{-/-}$* or wildtype (*$P2Y_2R^{+/+}$ / $Enpp1^{+/+}$)* cell lysates using a colorimetric assay (Anaspec, CA, USA), as previously described (Orriss et al., 2012a). TNAP activity was normalised to cell protein using the Bradford assay.

Histology

Histological analysis was performed on aortas obtained from 24-week old male *$P2Y_2R^{+/+}$* or *$P2Y_2R^{-/-}$* mice. Tissues were fixed in 10% neutral buffered formalin and embedded in paraffin wax blocks. Serial

sections were cut every 5µm and mounted onto slides. Before staining, the samples were de-paraffinised using xylene, then rehydrated through a series of decreasing ethanol solutions and finally water. Slides were stained with haematoxylin and eosin (H&E) to examine cell morphology and alizarin red to visualise vascular calcification.

Statistical analysis

Data were analysed using GraphPad Prism 6 software (San Diego, CA). Statistical comparisons were made using one-way or two-way analysis of variance (ANOVA) with a post-hoc Bonferroni correction for multiple comparisons. Results are expressed as means ± SEM for 6 replicates and are representative of experiments performed at least three times using cells or tissues obtained from different animals.

RESULTS

P2 receptor expression is increased in calcifying VSMCs

Analysis of mRNA expression revealed that mouse VSMCs express multiple P2 receptor subtypes including all the P2X receptors and the majority of the P2Y receptors **(Fig. 1A)**. Expression of the P2X1, P2X2, P2X4, P2X5, P2X6 and $P2Y_2$ receptors was increased up to 3-fold in calcifying VSMCs compared to control VSMCs (**Fig.1A**).

The relative level of P2 receptor mRNA expression between calcifying VSMCs and mineralising osteoblasts was also investigated (**Fig. 1B**). Expression of P2X3 receptor mRNA was increased 2.5-fold in calcifying VSMCs. In contrast, levels of the P2X5, P2X6, P2X7, $P2Y_1$, $P2Y_2$ and $P2Y_{13}$ receptors were reduced in calcifying VSMCs compared to mineralising osteoblasts (**Fig. 1B**). All other P2 receptors was displayed a similar level of expression

ATP, UTP and PP_i inhibit VSMC calcification

ATP (≥10μM) and UTP (≥1μM) dose dependently inhibited VSMC calcification by up to 80% (**Fig. 2A, 2B, 2F).** UTP was more potent, exerting inhibitory actions from 1μM. ADP and UDP had no effect at any of the concentrations tested **(Fig. 2C, 2D, 2F**). The ubiquitous mineralisation inhibitor, PP_i (≥10μM), reduced VSMC calcification up to 85% **(Fig. 2E, 2F).** Representative images in **Fig. 2F** show the inhibitory actions of 10μM ATP, UTP or PP_i on calcification in mouse VSMC cultures.

ATP, UTP and PP_i increase VSMC viability and decrease apoptosis

In calcifying VSMCs, treatment with ATP, UTP and PP_i (≥10μM) decreased the percentage of dead cells present by up to 75%, 60% and 80%, respectively; ADP and UDP had no effect **(Fig. 3A-3E).** ATP, UTP, ADP and UDP had no effect on osteoblast cell viability **(Fig. 3F-3I).** PP_i at the highest dose (100μM) caused a 45% increase in the proportion of dead cells **(Fig. 3J)**.

Apoptosis was increased up to 2.3-fold in calcifying VSMCs compared to control VSMCs **(Fig 3K-3M)**. Treatment with ATP, UTP and PP_i (≥10μM) decreased the level of apoptosis in calcifying cells to the level seen in control VSMCs **(Fig 3K-3M)**.

The effect of ATP, UTP and PP_i on TNAP activity in calcifying VSMCs and osteoblasts

ATP and UTP (≥1μM) increased VSMC TNAP activity by up to 88% and 50%, respectively (**Fig. 4A, 4B**). PP_i, (≥1μM), which is a substrate for TNAP, dose dependently stimulated enzyme activity by up to 80% **(Fig. 4C)**. In contrast, ATP and UTP (≥1μM) inhibited osteoblast TNAP activity by up to 50% **(Fig. 4D, 4E).** PPi had no effect on TNAP activity in osteoblasts (**Fig. 3F).** It should also be noted that the basal TNAP activity of mineralising osteoblasts was at least 100-fold higher than that of a calcifying VSMCs **(Fig. 4).**

The $P2Y_2$ receptor does not mediate the effects of ATP & UTP on VSMC calcification

Deletion of the $P2Y_2$ receptor had no effect on the basal level of calcification in VSMC or aortic ring cultures **(Fig. 5A, 5B)**. The inhibitory effects of ATP and UTP on calcification were unchanged in $P2Y_2R^{-/-}$ VSMCs **(Fig. 5C, 5D).** The selective $P2Y_2$ receptor antagonist, ARC118935XX, also failed to attenuate the inhibitory effects of UTP **(Fig. 5E)**. Isolated aortas from 24-week old $P2Y_2R^{+/+}$ and $P2Y_2R^{-/-}$ mice showed no obvious differences in structure or cell morphology **(Fig. 5F**). Alizarin red staining revealed no spontaneous calcium deposition within any of the arterial layers **(Fig. 5F**). Representative images shown are from the descending aorta. The effects of ATP and UTP on VSMC cell viability and TNAP activity were also unaffected by the deletion of the $P2Y_2$ receptor **(Fig. 5G-5J).**

Reduced inhibitory effects of ATP and UTP on bone mineralisation in $P2Y_2R^{-/-}$ osteoblasts

ATP (≥10μM) and UTP (≥1μM) dose dependently reduced bone mineralisation in $P2Y_2R^{+/+}$ osteoblasts by up to 80% (**Fig. 6A, 6B, 6E**). The overall level of mineralised bone nodule formation was up to 60% higher in $P2Y_2R^{-/-}$ cells compared to $P2Y_2R^{+/+}$. In $P2Y_2R^{-/-}$ osteoblasts, the effects of ATP and UTP were

only observed at 100μM making them 10-fold and 100-fold less potent at inhibiting bone mineralisation, respectively (**Fig. 6A, 6B, 6E**). Representative whole well scans (unstained) and phase contrast microscopy images (alizarin red stained) of osteoblast cell layers show the reduced inhibitory effects of UTP in *P2Y$_2$R$^{-/-}$* osteoblasts. They also illustrate the increased level of bone formation in *P2Y$_2$R$^{-/-}$* cells **(Fig. 6E).**

The inhibitory effects of ATP and UTP on TNAP activity are lost in P2Y$_2$R$^{-/-}$ osteoblasts

In *P2Y$_2$R$^{+/+}$*osteoblasts ATP (≥10μM) and UTP (≥1μM) reduce TNAP activity by up to 40%**.** *P2Y$_2$R$^{-/-}$* osteoblasts display basal TNAP activity levels that are up to 30% higher than wildtype cells. The inhibitory effects of ATP and UTP on TNAP activity were lost in *P2Y$_2$R$^{-/-}$* osteoblasts **(Fig. 6C, 6D)**.

The inhibitory effects of ATP and UTP on VSMC calcification and bone mineralisation are reduced in Enpp1$^{-/-}$ mice

Since PP_i mimics many of the actions of ATP and UTP we investigated whether the effects of these extracellular nucleotides involved P2 receptor independent mechanisms. In *Enpp1$^{+/+}$* cells, ATP and UTP inhibit VSMC calcification from concentrations of 10μM and 1μM, respectively. In *Enpp1$^{-/-}$* VSMCs, ATP and UTP are 10-fold less potent only exerting inhibitory effects at 100μM and 10μM, respectively **(Fig. 7A, 7B).**

Consistent with earlier studies, *Enpp1$^{-/-}$* osteoblasts displayed increased levels of bone mineralisation compared to *Enpp1$^{+/+}$* cells (Anderson et al., 2005; Orriss et al., 2015). The inhibitory effects of ATP and UTP on bone mineralisation were 10-fold and 100-fold less potent, respectively, in *Enpp1$^{-/-}$* osteoblasts **(Fig. 7C, 7D).** Representative whole well scans (unstained) and phase contrast microscopy images (alizarin red stained) of osteoblast cell layers show the reduced inhibitory effects of ATP in *Enpp1$^{-/-}$* osteoblasts (**Fig. 7E).**

NPP1 deletion does not blunt the effects of ATP and UTP on cell viability and TNAP activity

The protective effects of ATP and UTP against loss of cell viability were unchanged in cultures of *Enpp1$^{-/-}$* VSMCs (**Fig. 8A, 8B).** Deletion of NPP1 also had no effect on the ATP/UTP-mediated increase in VSMC TNAP activity **(Fig. 8C, 8D**).

DISCUSSION

This study compared the effects of extracellular nucleotides on AMC and bone mineralisation. We found that, whilst the functional effect was the same, the cellular mechanisms mediating the observed inhibitory actions of ATP and UTP differed. In VSMCs, ATP and UTP prevent calcification, at least in part, by reducing VSMC apoptosis. In contrast, ATP and UTP have no effect on osteoblast survival and instead block bone mineralisation via the inhibition of TNAP. Furthermore, the $P2Y_2$ receptor does not appear to mediate the actions of ATP and UTP on AMC but is involved in the effects on bone mineralisation. However, NPP1-mediated generation of PP_i contributes to the inhibitory effects observed in both VSMCs and osteoblasts (see summary **Fig. 9**).

This study demonstrated, like others, that VSMCs express multiple P2X and P2Y receptor subtypes (Lewis and Evans, 2000; Wang et al., 2002). Interestingly, the mRNA expression of several P2 receptors was upregulated in calcifying VSMCs compared to control cells. Since the expression of P2 receptors by osteoblasts is differentiation-dependent (Dixon et al., 1997; Noronha-Matos et al., 2012; Orriss et al., 2006), this could reflect the changes in the VSMC phenotype which occur during the development of AMC.

Consistent with earlier work that described protective effects of extracellular nucleotides on aortic valve calcification (Cote et al., 2012) and VSMC calcification (Villa-Bellosta and Sorribas, 2013), we also found that both ATP and UTP were inhibitory. Whereas, ADP and UDP were without effect. UTP, which was more potent than ATP, only activates the $P2Y_2$ and $P2Y_4$ receptor subtypes. Given the involvement of the $P2Y_2$ receptor in inhibiting bone mineralisation (**Fig. 6**) (Hoebertz et al., 2002; Orriss et al., 2007), aortic valve calcification and intimal calcification (Cote et al., 2012; Qian et al., 2017), the $P2Y_2$ receptor appeared to be a strong candidate for mediating the effects in VSMCs. However, the effects of ATP and UTP on VSMC calcification were not lost in *$P2Y_2R^{-/-}$* cells or blocked by a selective $P2Y_2$ receptor antagonist. Furthermore, *$P2Y_2R^{-/-}$* VSMCs and aortic rings displayed no differences in the basal level of calcification *in vitro* and isolated aortas from 24-week old animals also showed no signs of AMC. It should be noted that aged animals were not studied here and so it is possible that the condition develops in older *$P2Y_2R^{-/-}$* mice. Taken together, these data suggest that ATP and UTP are not acting via the $P2Y_2$ receptor to regulate AMC.

Our findings contrast to previous work which has implicated the $P2Y_2$ receptor in the effects of nucleotides on valve calcification (Cote et al., 2012) and arterial intimal calcification (Qian et al., 2017). There are a number of potential explanations for these observed differences. First, the lack of involvement of the $P2Y_2$ receptor in aortic VSMCs could represent variation in the cellular mechanisms that underpin these different forms of pathological vascular calcification. Second, it could be due to differences in the experimental models used since each investigation has utilised distinct *in vitro* and *in vivo* methods to study the different forms of vascular calcification. Finally, we observed that $P2Y_2$ receptor mRNA expression was significantly lower in calcifying VSMCs than mineralising osteoblasts. This suggests that $P2Y_2$ receptor-mediated signalling could be of lesser importance in the regulation of calcification processes in aortic VSMCs.

Extracellular nucleotides are rapidly hydrolysed by ecto-nucleotidases, meaning that the parent molecule is only present in the culture medium for a very short period of time. Particularly important is the breakdown of ATP and UTP by NPP1 to produce the key local inhibitor of mineralisation, PP_i. Since the effects of PP_i on VSMC calcification mimicked those of ATP and UTP, we used *$Enpp1^{-/-}$* cells to determine the involvement of P2 receptor-independent mechanisms. The effects of ATP and UTP were blunted in *$Enpp1^{-/-}$* VSMCs and osteoblasts suggesting that NPP1-mediated hydrolysis to produce PP_i does contribute to the observed inhibitory actions in both cell types. This is consistent with earlier work that examined the role of ATP-derived PP_i in VSMC calcification (Prosdocimo et al., 2009; Prosdocimo et al., 2010) and bone formation (Orriss et al., 2013; Orriss et al., 2007). However, since the effects were not completely attenuated it suggests that other mechanisms are also involved. In osteoblasts, this is

likely to involve activation of the P2 receptors implicated in the regulation of bone mineralisation by ATP and/or UTP, such as the $P2Y_2$, P2X1 and P2X7 receptors (Hoebertz et al., 2002; Orriss et al., 2012a; Orriss et al., 2007). In VSMCs, the other mechanisms leading the functional effects are less clear. Although unlikely to be mediated by the $P2Y_2$ receptor, involvement of other P2 receptor subtypes (e.g. the $P2Y_4$ receptor) is probable, and presents an area for future study. Furthermore, since VSMCs have been shown to also express functional NPP3 (Prosdocimo et al., 2009), a role for other ecto-nucleotidases cannot be discounted.

Increased VSMC apoptosis has been implicated in the development of vascular calcification (Proudfoot et al., 2000). In this study we found that, at the concentrations which inhibited calcification, ATP, UTP and PP_i prevented the increase in apoptosis usually seen in calcifying VSMC cultures. The effect of extracellular nucleotides on cell viability was unchanged in *Enpp1*$^{-/-}$ VSMCs. This suggests that, although PP_i itself appears to exert protective actions, ATP and UTP are working directly to promote VSMC survival in calcifying conditions. Apoptosis is thought to promote calcification because the VSMC-derived apoptotic bodies can act as a nucleation site for hydroxyapatite crystal formation (Proudfoot et al., 2000). Thus, the inhibitory effects of extracellular nucleotides (and PP_i) on AMC could be mediated, at least in part, by their ability to reduce apoptosis. The overall result being that there are less apoptotic bodies on which calcification can be initiated. In contrast, extracellular nucleotides have no effect on osteoblast viability and so the inhibitory effects on bone mineralisation are clearly not mediated via alterations in apoptosis.

These differential effects of extracellular nucleotides on cell survival may also illustrate the differences between physiological bone mineralisation and pathological AMC. Bone mineralisation is not associated with increased levels of osteoblast apoptosis and instead matrix vesicles are the primary nucleation site for hydroxyapatite formation (Anderson et al., 1975). The initiation of AMC appears more heterogeneous with multiple factors acting as nucleation sites for calcification including apoptotic bodies and mineralisation-competent vesicles (Kapustin and Shanahan, 2012; Proudfoot et al., 2000).

TNAP is a crucial enzyme in bone mineralisation, with deficiencies leading to hypophosphatasia, a condition characterised by hypomineralisation and rickets (Caswell et al., 1991). Increased TNAP expression and activity has also been associated with AMC (Narisawa et al., 2007; Sheen et al., 2015). We have previously shown that ATP and UTP reduce TNAP expression and activity in osteoblasts (Orriss et al., 2007). Here, we demonstrate that these inhibitory actions involve $P2Y_2$ receptor activation since the effects on TNAP activity were lost in *P2Y$_2$R*$^{-/-}$ osteoblasts. In contrast, at concentrations where calcification is inhibited by up to 80%, ATP and UTP stimulated VSMC TNAP activity. The reasons for this counterintuitive increase are unclear but since PP_i, and to a lesser extent ATP and UTP, are hydrolysed by TNAP the higher substrate levels could lead to alterations in enzyme kinetics. It is important to note that, even when stimulated, the TNAP activity of calcifying VSMCs was at least 100-fold lower than that of mineralising osteoblasts *in vitro*. Taken together, our observations suggest that whilst reduced TNAP activity contributes to the inhibition of bone mineralisation by ATP and UTP, alterations in TNAP activity are unlikely to mediate the functional effects of nucleotides on VSMC calcification. Instead the effects on VSMC apoptosis appear to predominate. Furthermore, although TNAP is essential for bone mineralisation its role in AMC appears more complex.

It should be noted that, whilst cell culture conditions were very similar, they were not identical because the use of different phosphate sources was required; VSMCs and osteoblasts were grown in 2mM sodium phosphate or 2mM β-glycerophosphate, respectively. VSMCs did not calcify in 2mM β-glycerophosphate probably because they lack sufficient TNAP to generate the free phosphate needed for calcification. Whilst it is unlikely that this small variation in culture conditions caused the different cellular responses to extracellular nucleotides, contributory effects cannot be fully discounted.

It is widely accepted that the development of AMC shares similarities to physiological bone formation. Whilst the role of NPP1 was similar in both VSMCs and osteoblasts, this study demonstrated that there

are also clear differences in the cellular mechanisms mediating the inhibitory effects of extracellular nucleotides. This suggests that some of the underlying processes leading to AMC and bone mineralisation are not the same. It is these differences which need to be fully understood in order to identify a drug target or therapeutic agent that can prevent or regress AMC without exerting negative actions on the skeleton.

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FIGURE LEGENDS

Figure 1. ***Expression of P2 receptors by calcifying VSMCs***

(A) VSMCs express mRNA for multiple P2 receptor subtypes; expression of the P2X1, P2X2, P2X4, P2X5, P2X6 and $P2Y_2$ receptors is increased up to 3-fold in calcifying cells compared to control cells. **(B)** Compared to mineralising osteoblasts, calcifying VSMCs show increased P2X3 receptor (2.5 fold) expression but reduced levels of the P2X5, P2X6, P2X7, $P2Y_1$, $P2Y_2$ and $P2Y_{13}$ receptors. Values are mean ± SEM (*n* = 4, * = p<0.05, ** = p<0.01, *** = p<0.001

Figure 2. ***The functional effects of extracellular nucleotides on VSMC calcification***

(A) ATP and **(B)** UTP dose dependently (≥1μM) inhibit VSMC calcification by up to 80%. **(C)** ADP and **(D)** UDP have no effect on VSMC calcification. **(E)** PP_i (≥10μM) reduces VSMC calcification by up to 85%. Values are mean ± SEM (*n* = 6), * = p<0.05, ** = p<0.01, *** = p<0.001. **(F)** Representative light microscopy images of alizarin red stained VSMC cultures treated with extracellular nucleotides. Scale bar = 50μm

Figure 3. ***ATP, UTP and PPi reduce VSMC cell death but have no effect on osteoblast survival***

(A) ATP and **(B)** UTP dose dependently decrease the proportion of dead cells in calcifying VSMC cultures by up to 75%; **(C)** ADP and **(D)** UDP have no effect. **(E)** PP_i reduced the level of dead cells by up to 80%. **(F, G, H, I)** ATP, UTP, ADP and UDP do not influence osteoblast viability. **(J)** PP_i at the highest dose tested caused a small increase in the percentage of dead cells. **(K, L, M**) Culture of VSMCs in calcifying conditions was associated by an increased level of apoptosis (up to 2.3 fold). ATP, UTP and PP_i (≥10μM) reduced the amount of apoptosis to levels seen in non-calcifying cells. Values are mean ± SEM (*n* = 6), * = p<0.05, ** = p<0.01, *** = p<0.001.

Figure 4. ***Opposing effects of extracellular nucleotides on TNAP activity in VSMCs and osteoblasts***

(A) ATP, **(B)** UTP and **(C)** PP_i (≥1μM) increased VSMC TNAP activity by up to 88%. **(D)** ATP and **(E)** UTP reduced osteoblast TNAP activity by up to 50%. **(F)** No effect of PP_i on osteoblast TNAP activity. Note that the basal levels of TNAP activity are approximately 100-fold higher in mineralising osteoblasts than calcifying VSMCs. Values are mean ± SEM (*n* = 6), * = p<0.05, ** = p<0.01, *** = p<0.001.

Figure 5. ***Lack of involvement of the $P2Y_2$ receptor in regulating AMC***

(A) Cultured VSMCs and **(B)** aortic rings from $P2Y_2R^{-/-}$ animals displayed no differences in the basal level of calcification. The inhibitory effects of **(C)** ATP and **(D)** UTP were unchanged in $P2Y_2R^{-/-}$ VSMCs. **(E)** The selective $P2Y_2$ receptor antagonist, ARC118925XX, did not block the inhibitory effects of UTP on VSMC calcification. **(F)** Histological analysis of isolated aortas from 24-week old $P2Y_2R^{-/-}$ mice showed no difference in cell morphology or the presence of AMC. Scale bar = 100μm. The protective effects of **(G)** ATP and **(H)** UTP on cell viability were unaffected by deletion of the $P2Y_2$ receptor. The stimulatory actions of **(I)** ATP and **(J)** UTP on TNAP activity were unchanged in $P2Y_2R^{-/-}$ VSMCs. Values are mean ± SEM (*n* = 6), * = p<0.05, ** = p<0.01, *** = p<0.001.

Figure 6. ***The role of the $P2Y_2$ receptor in the inhibitory effects of ATP and UTP on bone mineralisation***

The inhibitory effects of **(A)** ATP and **(B)** UTP were 10-fold and 100-fold less potent, respectively, in $P2Y_2R^{-/-}$ osteoblasts; mineralised bone nodule formation was 60% higher in $P2Y_2R^{-/-}$ osteoblasts. The inhibitory actions of **(C)** ATP and **(D)** UTP on TNAP activity were lost in $P2Y_2R^{-/-}$ osteoblasts. Basal TNAP activity was increased up to 40% in $P2Y_2R^{-/-}$ cells. Values are mean ± SEM (*n* = 6), * = p<0.05, ** = p<0.01, *** = p<0.001. Comparison of $P2Y_2R^{+/+}$ to $P2Y_2R^{-/-}$: # = p<0.05, ## = p<0.01, ### = p<0.001. **(E)** Representative whole well scans (unstained) and phase contrast microscopy images (alizarin red stained) showing the effects of UTP on bone mineralisation in $P2Y_2R^{-/-}$ and $P2Y_2R^{+/+}$ osteoblasts. Areas

of unmineralised matrix are highlighted by the arrows. Scale bars: whole well scans = 0.5cm, microscopy images = 200µm.

Figure 7. ***The actions of NPP1 contribute towards the inhibitory effects of ATP and UTP on VSMC calcification and bone mineralisation***

The inhibitory effects of **(A)** ATP and **(B)** UTP on calcification were 10-fold less potent in *Enpp1*$^{-/-}$ VSMCs. In *Enpp1*$^{-/-}$ osteoblasts **(C)** ATP and **(D)** UTP were 10-fold and 100-fold less potent, respectively, at blocking bone mineralisation. Values are mean ± SEM (*n* = 6), * = p<0.05, ** = p<0.01, *** = p<0.001. Comparison of *Enpp1*$^{+/+}$ to *Enpp1*$^{-/-}$: # = p<0.05, ### = p<0.001. **(E)** Representative whole well scans (unstained) and phase contrast microscopy images (alizarin red stained) showing the effects of ATP on bone mineralisation in *Enpp1*$^{+/+}$ and *Enpp1*$^{-/-}$ osteoblasts. Scale bars: whole well scans = 0.5cm, microscopy images = 200µm.

Figure 8. T*he effect of ATP and UTP on VSMC survival and TNAP activity does not involve NPP1*

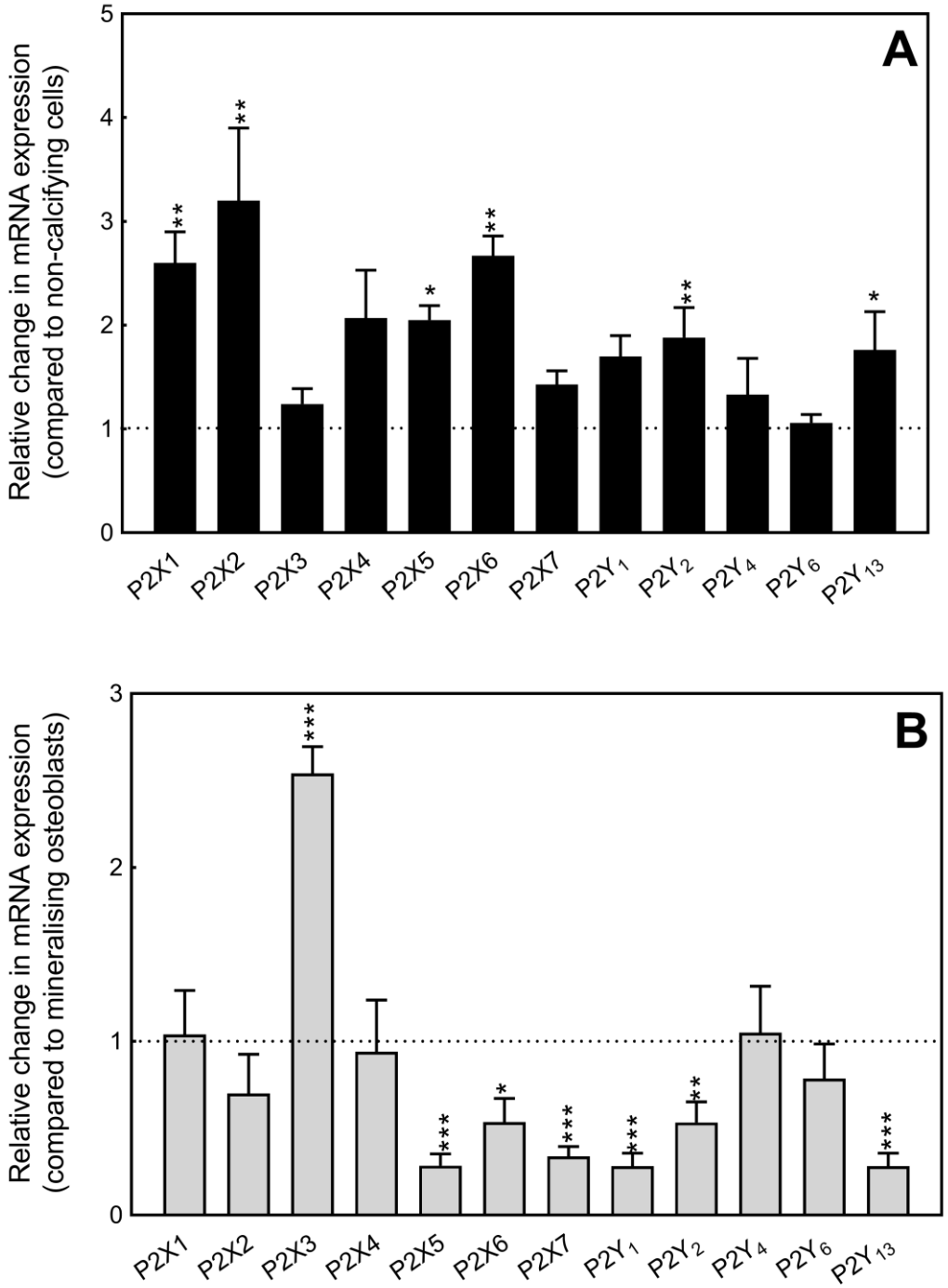
The protective effects of **(A)** ATP and **(B)** UTP on cell viability were unchanged in *Enpp1*$^{-/-}$ VSMCs. The stimulatory actions of **(C)** ATP and **(D)** UTP on TNAP activity were the same in *Enpp1*$^{+/+}$ and *Enpp1*$^{-/-}$ cells. Values are mean ± SEM (*n* = 6), * = p<0.05, *** = p<0.001.

Figure 9*. Summary of the differing cellular mechanisms by which ATP and UTP exert their effects on AMC and bone mineralisation*

ATP and UTP inhibit both AMC and bone mineralisation but there are some differences in the mechanisms mediating these actions. In VSMCs (top), ATP and UTP act via P2 receptors to decrease apoptosis and, as a consequence, calcification. In osteoblasts (OBs, bottom panel), ATP and UTP act via the $P2Y_2$ receptor to inhibit alkaline phosphatase (TNAP) activity. However, for both cell types hydrolysis of ATP and UTP to produce PP_i by NPP1 also contributes to the inhibitory effects observed.

Table 1: Primer sequences for real time PCR

Gene	Primer sequence (5'-3')	
$P2X_1$	***S***	tgt acg ggg aga aga acc tg
	AS	tcc caa aca cct tga aga gg
$P2X_2$	***S***	cgt ctt cat cgt gca gaa aa
	AS	cac ttt gtg ttc cga cat gg
$P2X_3$	***S***	tac caa gtc ggt ggt tgt ga
	AS	cca ccc cac aaa gta gga ga
$P2X_4$	***S***	gca ccc tcc acc atc tct aa
	AS	aaa cct ctt gcc aga agc aa
$P2X_5$	***S***	ggg ctt tct tct gtg acc tg
	AS	gtt ggc ctc aac ctc aac at
$P2X_6$	***S***	agc cat ggc ata aaa act gg
	AS	gtg aag ttc ttg gcc tga gc
$P2X_7$	***S***	ggc act gga gga aaa ttt ga
	AS	tga gca agt caa tgc aca ca
$P2Y_1$	***S***	agg aaa gct tcc agg agg ag
	AS	cgt gtc tcc att ctg ctt ga
$P2Y_2$	***S***	gtc agc agt gac gac tca aga c
	AS	tca gag gat atc agc ccc ttt a
$P2Y_4$	***S***	agg aag cag cag aac acc at
	AS	caa gga gtc tgc act ggt ca
$P2Y_6$	***S***	ttc cat ctt gca tga gac aga
	AS	gct tga aat cct cac ggt aga
$P2Y_{13}$	***S***	ggc cac tag atg tca cct ttt c
	AS	gat ggt ggg gtg gta act aga a


A
Relative change in mRNA expression
(compared to non-calcifying cells)
0
1
2
3
4
5
**
**
*
**
**
*
P2X1
P2X2
P2X3
P2X4
P2X5
P2X6
P2X7
P2Y1
P2Y2
P2Y4
P2Y6
P2Y13
B
Relative change in mRNA expression
(compared to mineralising osteoblasts)
0
1
2
3
***
***
*
***
***
**
***
P2X1
P2X2
P2X3
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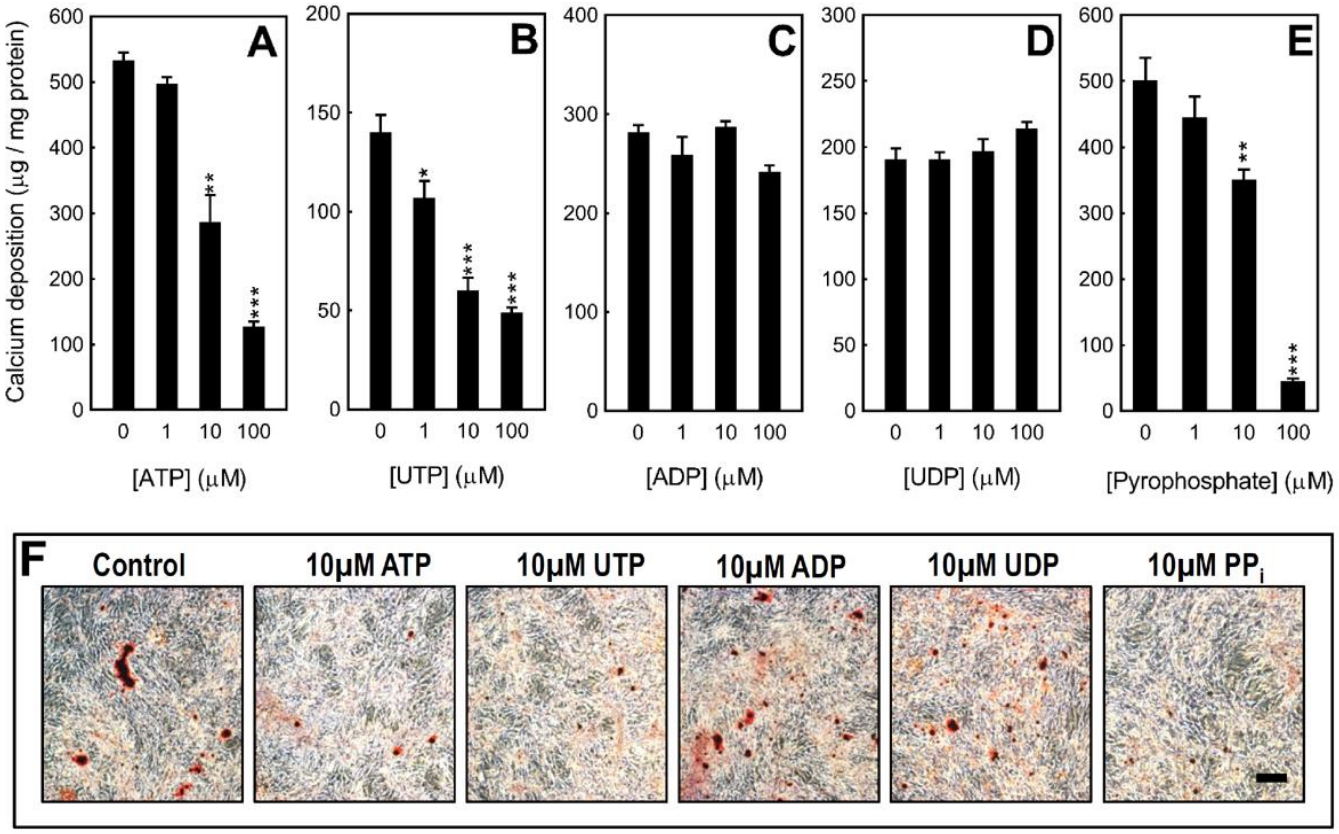
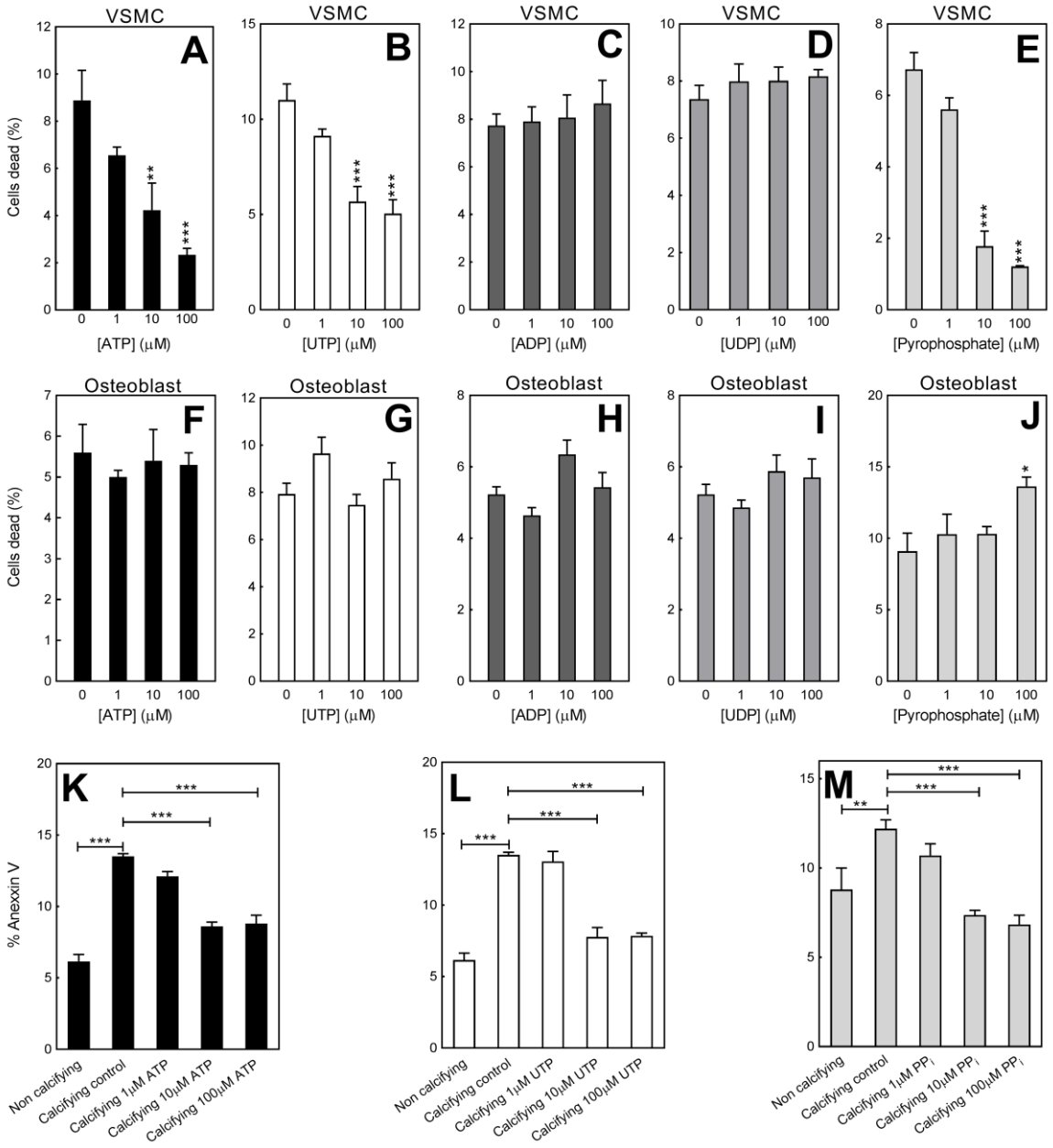
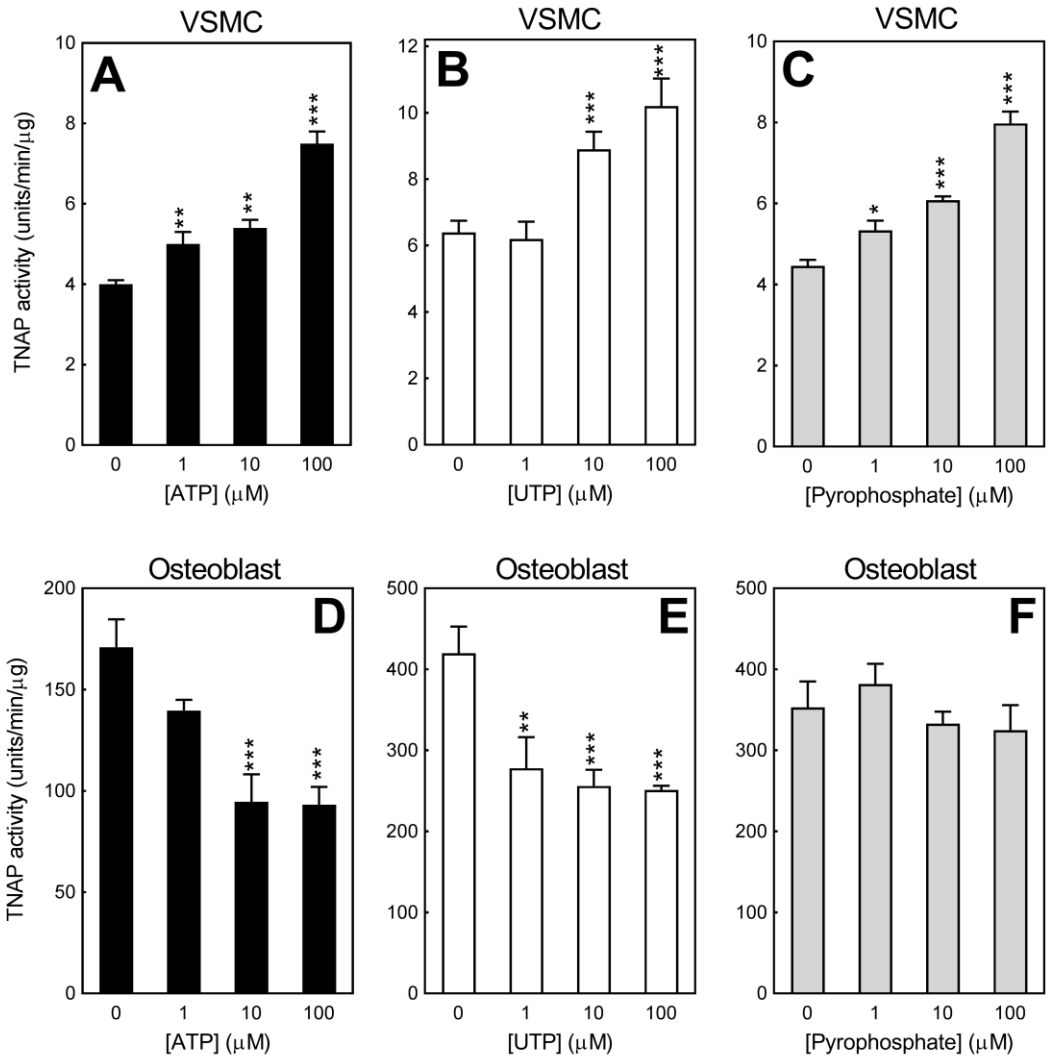
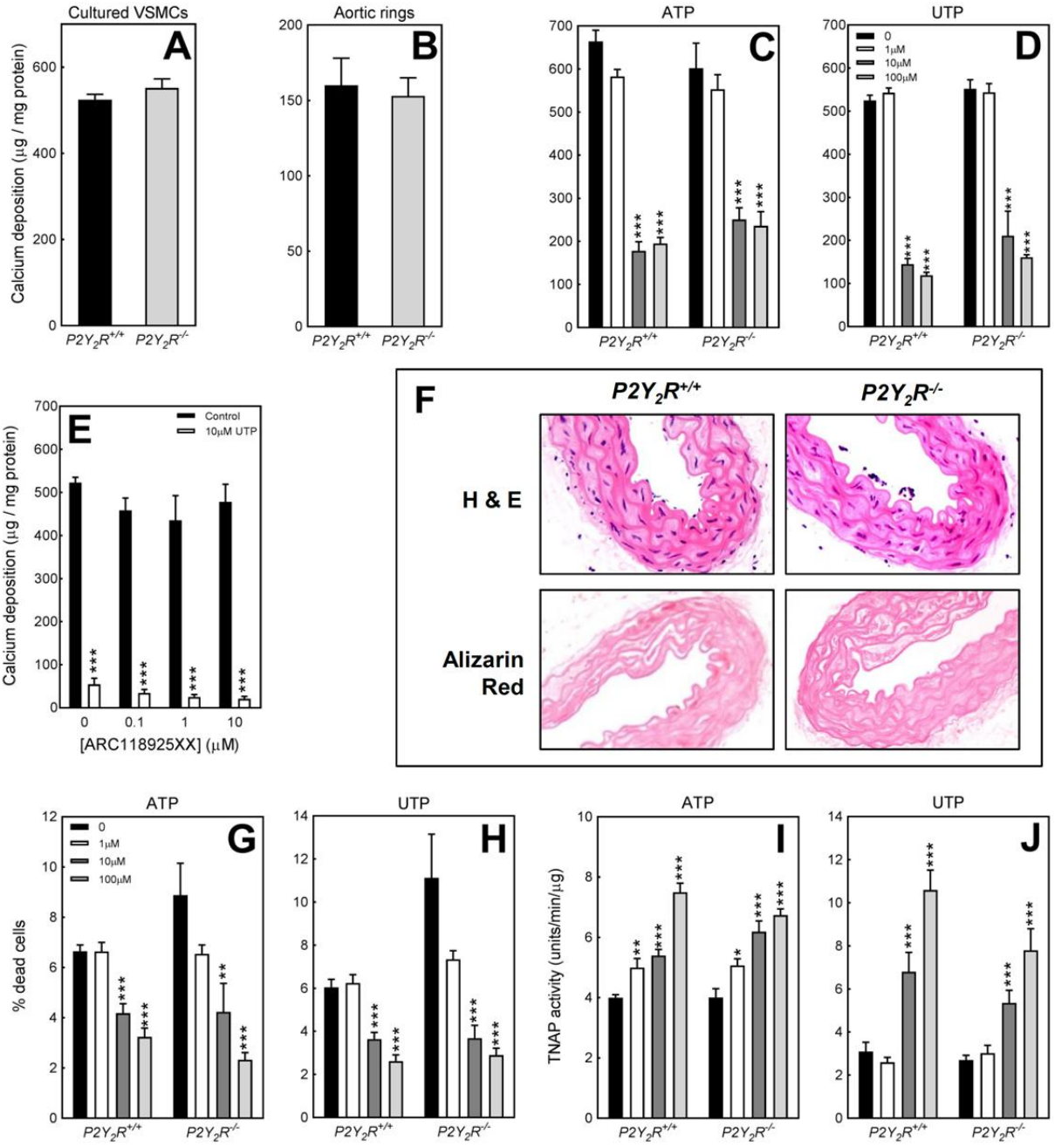
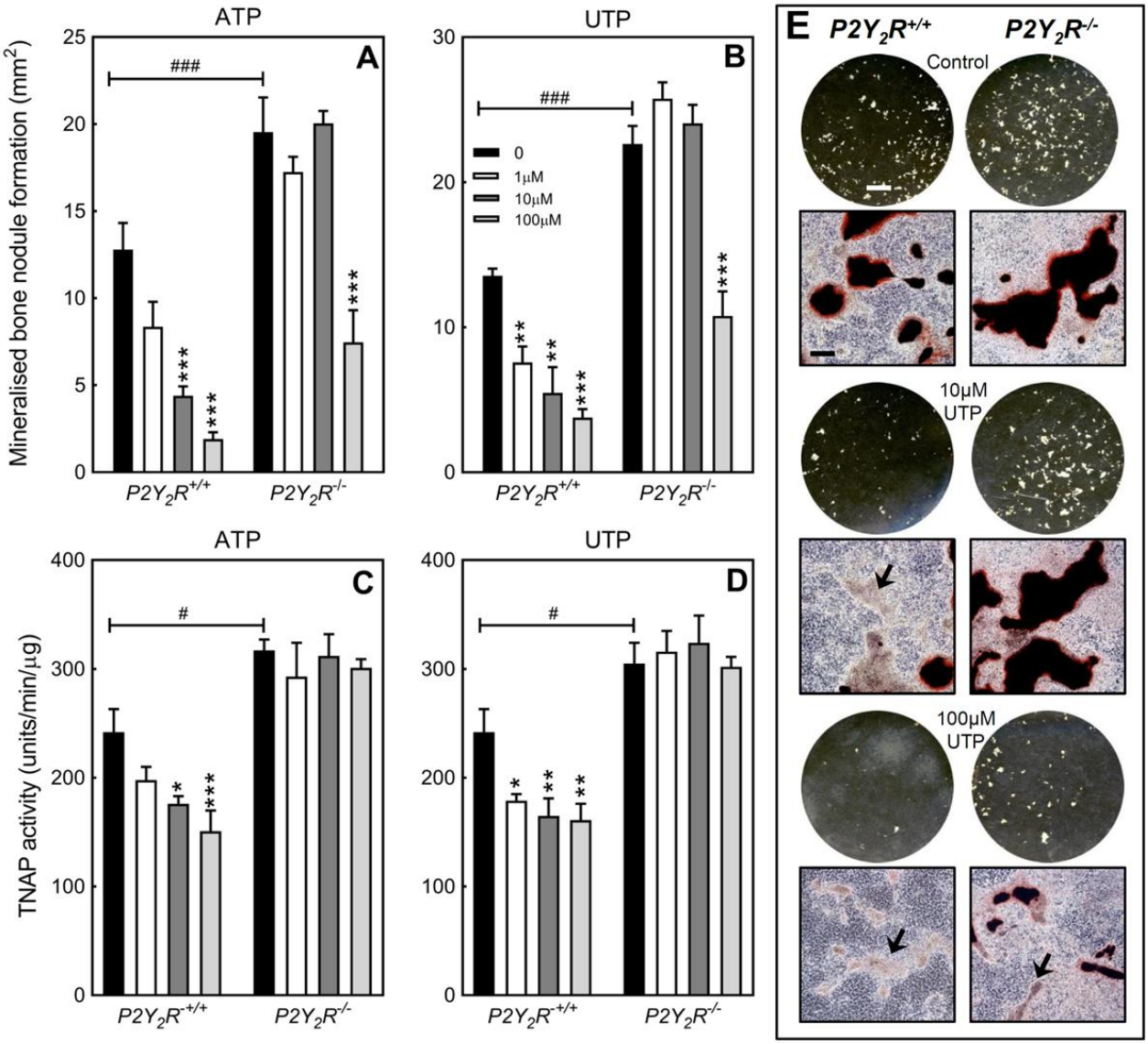
P2Y1
P2Y2
P2Y4
P2Y6
P2Y13

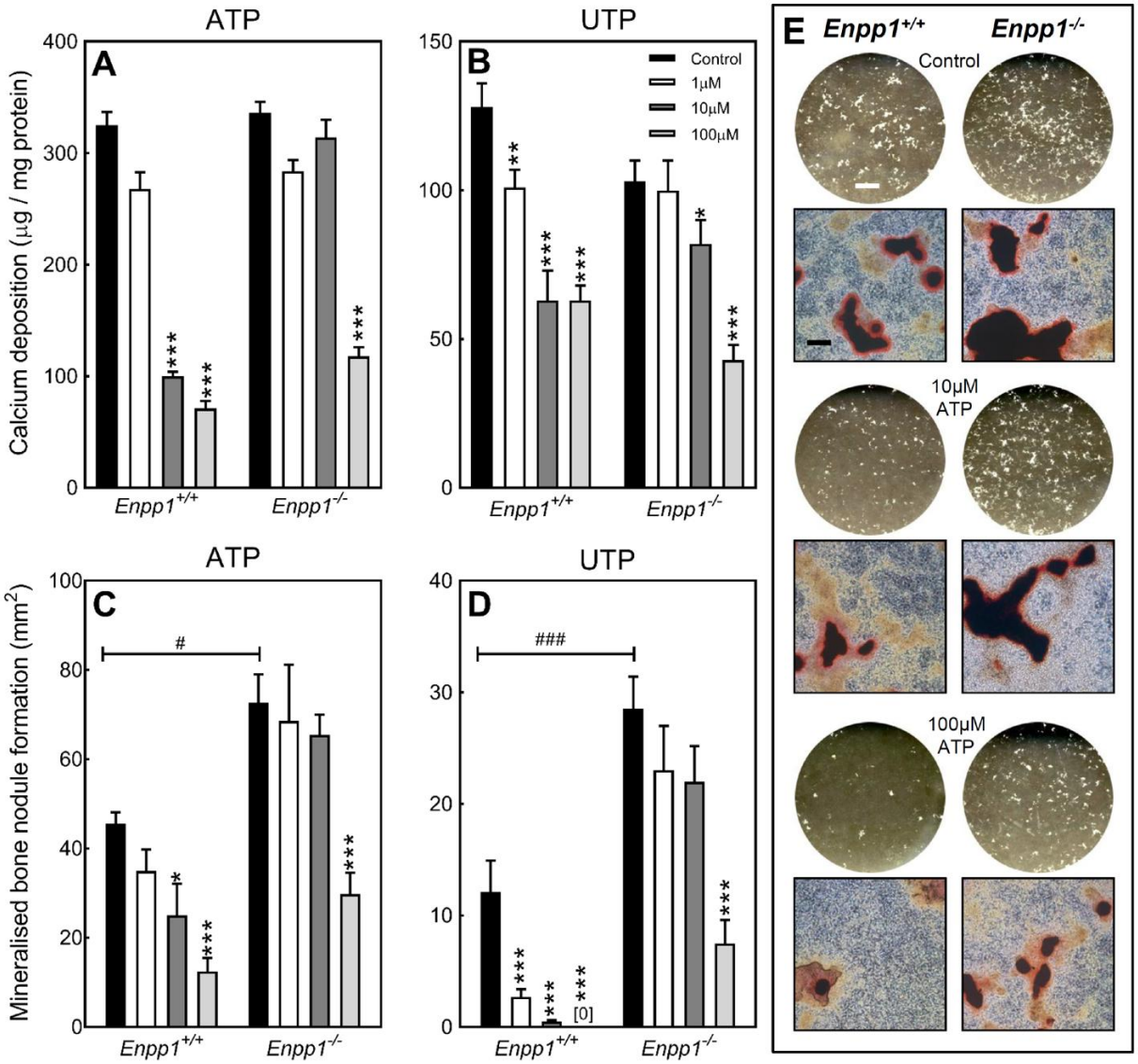

A
B
C
D
E
F
Calcium deposition (μg / mg protein)
[ATP] (μM)
[UTP] (μM)
[ADP] (μM)
[UDP] (μM)
[Pyrophosphate] (μM)
Control
10μM ATP
10μM UTP
10μM ADP
10μM UDP
10μM PPi

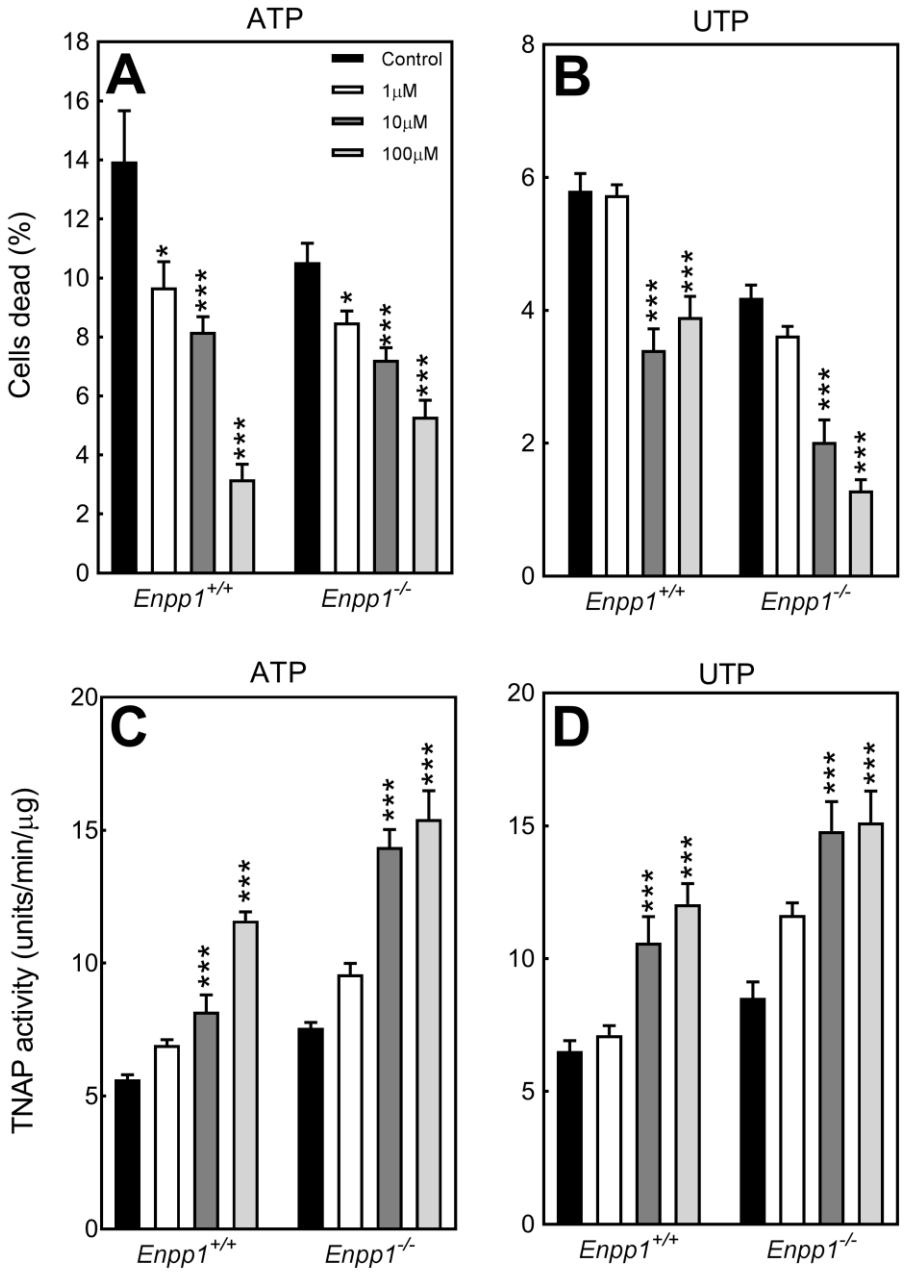

VSMC
A
B
C
D
E
Cells dead (%)
[ATP] (μM)
[UTP] (μM)
[ADP] (μM)
[UDP] (μM)
[Pyrophosphate] (μM)
Osteoblast
F
G
H
I
J
K
L
M
% Anexxin V
Non calcifying
Calcifying control
Calcifying 1μM ATP
Calcifying 10μM ATP
Calcifying 100μM ATP
Calcifying 1μM UTP
Calcifying 10μM UTP
Calcifying 100μM UTP
Calcifying 1μM PPi
Calcifying 10μM PPi
Calcifying 100μM PPi

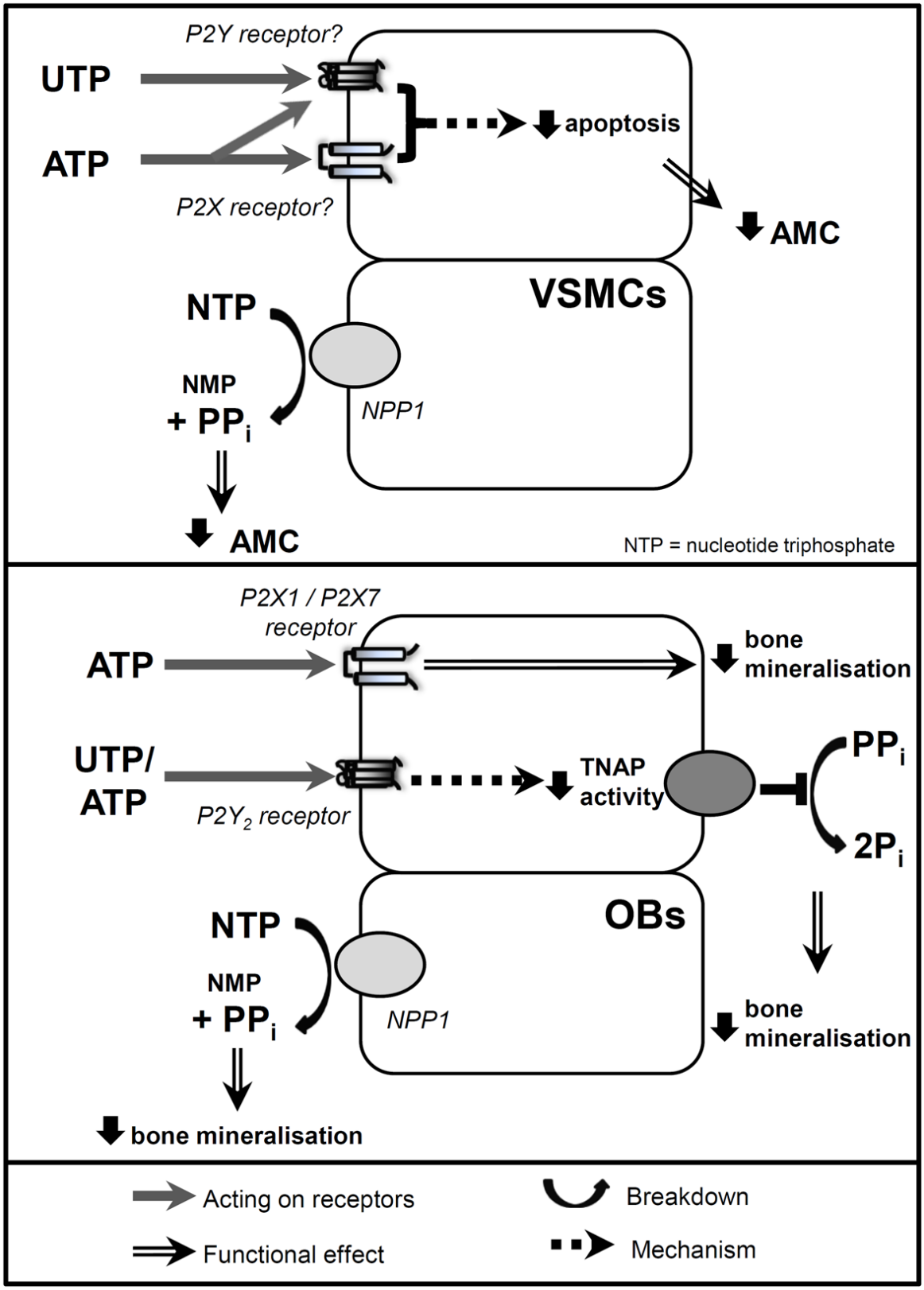

VSMC
A
B
C
TNAP activity (units/min/μg)
[ATP] (μM)
[UTP] (μM)
[Pyrophosphate] (μM)
Osteoblast
D
E
F
0
1
10
100
*
**
***


Cultured VSMCs
A
Calcium deposition (μg / mg protein)
P2Y2R+/+
P2Y2R-/-
Aortic rings
B
ATP
C
UTP
D
0
1μM
10μM
100μM
E
Control
10μM UTP
[ARC118925XX] (μM)
F
P2Y2R+/+
P2Y2R-/-
H & E
Alizarin Red
G
% dead cells
H
I
TNAP activity (units/min/μg)
J


ATP
UTP
A
B
C
D
E
Mineralised bone nodule formation (mm2)
TNAP activity (units/min/µg)
###
#
0
1µM
10µM
100µM
P2Y2R+/+
P2Y2R-/-
P2Y2R-+/+
P2Y2R-/-
Control
10µM UTP
100µM UTP


ATP
UTP
A
B
C
D
E
Calcium deposition (μg / mg protein)
Mineralised bone nodule formation (mm²)
Control
1μM
10μM
100μM
Enpp1+/+
Enpp1-/-
Enpp1+/+
Enpp1-/-
Control
10μM ATP
100μM ATP
[0]


ATP
UTP
A
B
C
D
Control
1μM
10μM
100μM
Cells dead (%)
TNAP activity (units/min/μg)
Enpp1+/+
Enpp1-/-
*
***


P2Y receptor?
UTP
ATP
P2X receptor?
apoptosis
AMC
VSMCs
NTP
NMP
+ PPi
NPP1
AMC
NTP = nucleotide triphosphate
P2X1 / P2X7
receptor
ATP
bone
mineralisation
UTP/
ATP
P2Y2 receptor
TNAP
activity
PPi
2Pi
OBs
NTP
NMP
+ PPi
NPP1
bone
mineralisation
bone mineralisation
Acting on receptors
Functional effect
Breakdown
Mechanism